[CANCER RESEARCH 52, 5379–5385, October 1, 1992]

Monovalent Immunotoxin Containing Truncated Form of *Pseudomonas* Exotoxin as Potent Antitumor Agent[1]

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ABSTRACT

Recombinant truncated forms of *Pseudomonas* exotoxin A that lack the cell binding domain of *Pseudomonas* exotoxin A were coupled to an F(ab') fragment of a monoclonal antibody HB21 directed against the human transferrin receptor. One of these was NlysPE40. The other, NlysPE38QQR, has two amino groups on residues near the NH_2-terminus and has no amino groups near the COOH-terminus. The proteins were linked by a stable thioether bond that connected the sulfhydryl group present in the hinge region of the antibody fragment to an amino group on the toxin. The F(ab')-PE40 immunotoxin, containing NlysPE40, exhibited potent cytotoxic activity on human carcinoma cell lines with a concentration of immunotoxin at which isotope incorporation falls by 50% when compared to nontreated cells (ID_{50}) of 5.3 pM (0.5 ng/ml) on both the epidermoid carcinoma A431 and on the colon carcinoma Colo205. Immunotoxins made with whole antibody were considerably less active, with an ID_{50} of 15.9 pM (3.1 ng/ml) on these cell lines. F(ab')-PE38QQR, the immunotoxin containing NlysPE38QQR, was found to be the most active agent with an ID_{50} of 1.05 pM (0.1 ng/ml) on A431 cells. The greater cytotoxicity of immunotoxins containing fragmented antibody was probably due to the higher binding affinity of F(ab') conjugates in comparison to whole antibody conjugates to the transferrin receptor. The increase in cytotoxic activity of the immunotoxin made with NlysPE38QQR than that with NlysPE40 may reflect selective coupling of the toxin through NH_2-terminal amino groups. The monovalent and divalent immunotoxins had dose-dependent antitumor effects on human epidermoid carcinoma xenografts in nude mice. A431 tumors completely regressed in all animals at a total dose of 105 pmol (10 μg) of F(ab')-PE38QQR and of 154 pmol (30 μg) of IgG-PE38QQR. Furthermore, the F(ab') immunotoxin was less toxic to mice than the conjugate containing IgG (840 pmol or 80 μg of total dose causing measurable adverse effects *versus* 208 pmol or 40 μg, respectively). Thus, a truncated *Pseudomonas* exotoxin A molecule coupled to the F(ab') fragment of an antibody is more active and less toxic in mice than an immunotoxin made with a whole antibody. Therefore, the therapeutic index for the monovalent immunotoxin is about four times better than that for the divalent immunotoxin.

INTRODUCTION

MAbs,[4] by virtue of their affinities toward defined molecules, are the subject of intense interest in the field of targeted therapy (1, 2). MAbs which bind to antigens that are expressed preferentially on cancer cells or certain differentiated cells (1, 3) have been utilized either without modification or as $F(ab')_2$ or F(ab') fragments (2). MAbs have been linked to chemicals, enzymes, radioisotopes, or toxins in order to visualize tumors or cause cell death (1, 2). Antibodies conjugated to toxins are termed ITs, and these agents are now being evaluated for their usefulness in the treatment of cancer and other diseases (4).

Our laboratory has been developing immunotoxins containing PE or mutant forms of this toxin (5). PE is a M_r 66,000 single-chain protein containing three disulfide bonds (6). PE is composed of three structural domains (I, II, III), and each of these domains performs at least one specific function (7, 8). Domain Ia binds to the PE receptor (8, 9); domain II is required for translocation (10, 11); and domain III catalyzes the ADP-ribosylation of elongation factor 2 which arrests protein synthesis in eukaryotic cells causing cell death (8). Prior to translocation, PE is cleaved after arginine 279 to produce a M_r 37,000 COOH-terminal fragment, which comprises all of domain III and a portion of domain II, that reaches the cytosol (10). ITs containing PE, or forms of PE that lack its cell binding domain (PE40), have been shown to possess high cytotoxic activities *in vitro* and to display prominent antitumor effects on solid tumor xenografts growing in nude mice (12–14). An additional lysine residue has been introduced near the amino terminus of PE40 (lysPE40, Ref. 12; NlysPE40, Ref. 13) which is believed to facilitate the reactivity of the toxin with cross-linking reagents in the conjugation reaction.

Despite the fact that the ITs containing PE or its derivatives are very active on cultured cells, they often bind to antigens with 3- to 10-fold less avidity than the unconjugated MAb (13–15). Diminished binding may be due to chemical alteration of the antigen binding site or the presence of the toxin at a site which, by steric hindrance, diminishes the contact between the antibody and the antigen. We have previously found that lysPE40 or NlysPE40 may be linked to either the heavy or light chain of a MAb in an IT (12).[5] Therefore, we support the idea that the physical presence of the toxin conjugated to an antibody makes binding of an IT less efficient than an antibody alone.

In an attempt to make an immunotoxin with higher activity, we have prepared an immunotoxin with a defined structure in which an amino group at the amino terminal of a truncated form of PE is coupled to a thiol group in a F(ab') fragment of an antibody. In this immunotoxin, (*a*) the toxin is linked to an antibody at a specific location on the heavy chain; (*b*) the site of coupling between these two proteins is removed as far as possible from the antibody combining site; and (*c*) the toxin is coupled to an antibody through residues on the toxin preceding the proteolytic cleavage site in domain II at position 279 (16) (Fig. 1). To ensure that coupling occurs at the amino end of the toxin, we also used a derivative of NlysPE40, NlysPE38QQR, in which all three lysines in domain III have been mutated to other residues (16).[6,7] Thus, in NlysPE38QQR, the two primary amino groups available for modification are located exclusively in domain II at or near the NH_2-terminal and upstream of the processing site (Fig. 1). NlysPE38QQR also has a deletion of amino acids 365–380 of PE which removes a disulfide bond in domain Ib that is not essential for activity and

Received 6/3/92; accepted 7/17/92.

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[1] Part of this work has been presented in a poster discussion session, "Immunology 8," at the 83rd Annual Meeting of the American Association for Cancer Research, San Diego, CA, May 20–23, 1992.

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[4] The abbreviations used are: MAb, monoclonal antibody; IT, immunotoxin; PE, *Pseudomonas* exotoxin A; SMCC, succinimidyl 4(*N*-maleimidomethyl)cyclohexan 1-carboxylate; 2-IT, 2-iminothiolane.

[5] Unpublished observation.

[6] W. Debinski and I. Pastan, manuscript in preparation.

[7] Y. Jinno, D. FitzGerald, and I. Pastan, unpublished observation.

produces a smaller molecule that may enter tumors more readily (17). Masuho *et al.* (18) and Fulton *et al.* (19) have reported the construction of an IT composed of ricin A chain linked by a disulfide bond to the F(ab') fragments. However, F(ab')-ITs had altered cytotoxic activities when compared to whole antibody ITs. In this paper, we report the construction of ITs composed of F(ab') linked to either NlysPE40 or NlysPE38QQR. These ITs bind better to cellular receptors and are several times more active than the whole MAb conjugates *in vitro* and are extremely potent antitumor agents *in vivo*.

MATERIALS AND METHODS

Preparation of F(ab') Fragments of HB21. HB21 is a monoclonal antibody (IgG1) which reacts with human transferrin receptors and has been used in several previous studies (12, 20). F(ab') fragments were generated from HB21 by using the Pierce Immunotoxin kit. Briefly, HB21 was dialyzed against 20 mM sodium acetate, pH 4.2, and concentrated to 2 to 4 mg/ml by using Centricon 10 (Amicon, NY) membranes. A slurry of the immobilized pepsin was added together with an appropriate volume of sodium acetate buffer and antibody solution. The sample was incubated with constant shaking at 37°C for 6 to 12 h. This time was sufficient to digest more than 90% of the MAb to $F(ab')_2$ and Fc fragments. The immobilized pepsin was pelleted with the help of a separator and the supernatant containing $F(ab')_2$, fragmented Fc, and remnants of an undigested IgG were loaded onto a Protein A column. $F(ab')_2$ fragments were recovered in the flow-through of the column. The fractions enriched in $F(ab')_2$ were either dialyzed against 0.1 M NaH_2PO_4, pH 6.0, using dialysis tubing with molecular weight cutoff of 50,000, or concentrated and washed 4 times with the same buffer on Centricon 30 membranes.

Conjugation of IgG or the F(ab') of HB21 to NlysPE40 or NlysPE38QQR. The construction and purification of the recombinant form of NlysPE40 and NlysPE38QQR are described elsewhere (13, 16).[6] The $F(ab')_2$ in 0.1 M NaH_2PO_4, pH 6.0, at 2 mg/ml was reduced to F(ab') fragments before conjugation. The reduction was achieved by treating $F(ab')_2$ fragments with 10 mM 2-mercaptoethylamine at 37°C for 1.5 h. The F(ab') fragments were separated from the reductant on a Sephadex G-25 column (PD10, Pharmacia, Piscataway, NJ) equilibrated with 0.1 M NaH_2PO_4/5 mM EDTA, pH 6.0. These conditions maintain the thiol groups in a reduced form. The recombinant PE molecules were reacted with SMCC (21). A 3-fold molar excess of SMCC was added to toxins in 0.1 M NaH_2PO_4/1 mM EDTA, pH 7.0, at room temperature for 1 h. SMCC was removed on a PD10 column equilibrated and eluted with NaH_2PO_4, pH 7.0. The IgG was modified with 2-IT (22), which like SMCC reacts with the ϵ-amino group of lysine residues and the α-amino group of the NH_2-terminal amino acid. The IgG was incubated with a 3-fold molar excess of 2-IT in 0.1 M NaH_2PO_4/1 mM EDTA, pH 8.0, at 37°C for 30 min; a PD10 column was used to separate 2-IT from the antibody. Fractions from PD10 columns containing IgG or F(ab') were mixed with fractions containing toxins and incubated for 16 h at 25 or 4°C. The conjugates of IgG were purified on Mono-Q and TSK columns, as previously described (12), and conjugates of F(ab') were purified by TSK only. Conjugates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were detected by staining with Coomassie blue or by immunoblot analysis, which was performed with the use of polyclonal antisera to PE.

Binding Studies. HB21 was labeled with ^{125}I by using the lactoperoxidase method as described (23). The IgG (6 μg) was mixed with 0.5 mCi of the isotope $Na^{125}I$. Labeled antibody was separated from the unreacted isotope on a PD10 column. Human serum albumin was added to the fractions collected from gel filtration to a final concentration of 0.2% (w/v). The binding ability of ^{125}I-HB21 was estimated according to a previously described protocol (24), with a slight modification. After the amount of IgG with preserved immunoreactivity had been established we performed the binding assays. The binding analysis was carried out by using standard saturation and displacement curve experiments. Briefly, colon carcinoma cell line cells, Colo205, were seeded at 2×10^5 cells/well in 24-well tissue culture plates. After 24 h, the plates were placed on ice and cells were washed with ice-cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} in 0.2% bovine serum albumin. Then, increasing concentrations of HB21 or HB21-ITs were added to cells for 30 min before incubation with a fixed amount of ^{125}I-HB21 for 1.5 h with constant agitation. After incubation, the cells were washed twice, lysed with 0.1 N NaOH, and the content of radioactivity was determined.

Protein Synthesis Inhibition Assay. The cytotoxicity of IgG-PE40 and F(ab')-PE40 as well as the conjugates containing NlysPE38QQR was tested on the colon carcinoma cell line Colo205 and the epidermoid carcinoma cell line A431. HB21-ITs activities were measured as an inhibition of [^{3}H] leucine incorporation into cells (8).

Treatment of Nude Mice Bearing a Human Cancer. A431 cells (3×10^6/mouse) were injected s.c. on day 0 into female nude mice. Groups of 4 to 6 mice started to receive immunotoxin i.p. on day 4 after tumor implantation and control mice received either the vehicle or the toxin mixed but not conjugated with the F(ab') fragments. Tumor size was measured with a caliper and tumor volumes were calculated as previously reported (12).

Protein concentration was determined by the Bradford assay with the Pierce (Rockford, IL) Coomassie blue G-250-based reagent.

RESULTS

In the present work, we engineered a MAb-HB21 and PE mutants in a manner to ensure predictable conjugation to produce an IT. We made F(ab') fragments from HB21 and used the sulfhydryl group positioned in the hinge region of the antibody to link the antibody with NlysPE40 or NlysPE38QQR (Fig. 1). This sulfhydryl group occurs at the carboxyl end of the antibody fragment and is removed far from the combining site (2). It is the only place to which the toxin can attach. Coupling to

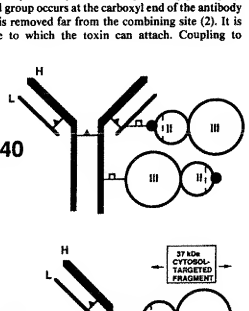

Fig. 1. Schematic representation of the (*A*) immunotoxin composed of MAb (*Y* letter simplified form) and NlysPE40 (domain II and III of PE symbolized by open circles); and (*B*) F(ab')-PE40 constructed by coupling of the reduced $F(ab')_2$ to NlysPE40. *Small closed circles* represent 11 amino acids which are placed in front of PE40 primary structure (Ref. 13). The *dashed line* in domain II symbolizes the cleavage site in PE40 [arginine 279 in PE; (10)]. Note that the linkage between domain III in NlysPE40 (*A* and *B*) and an antibody is possible; however, NlysPE38QQR can only be coupled to an antibody through either methionine or an additional lysine residue within 11-amino acid peptide whose location precedes the cleavage site of the toxin (see text). *L*, light chain; *H*, heavy chain; ▲, disulfide bond; ⌐⌐, thioether bond.

NlysPE40 can occur through the amino group on the NH_2-terminal methionine or lysine residue present in the 11-amino acid sequence preceding domain II (Fig. 1) or at positions 590, 606, and 613 of PE which are all located in domain III (16). NlysPE38QQR can be conjugated to an antibody only through the NH_2-terminal methionine or the lysine residue located in the 11-amino acid peptide sequence preceding domain II (Fig. 1).

Preparation and Analysis of ITs. HB21 was digested with pepsin and produced the expected $F(ab')_2$ fragments which were recovered from a Protein A column with an efficiency of 85.7 ± 1.5% (SD; $n = 3$) (Table 1; Fig. 2). After purification on the Protein A column, the $F(ab')_2$ was reduced with 2-mercaptoethylamine as described in "Materials and Methods." This produced F(ab') molecules, which had three thiol groups as detected by using Ellman's reagent. The IgG, when subjected to treatment with a thiolating agent at a 3-fold molar excess, had 0.4 thiols/mol. NlysPE40 treated with a 3-fold molar excess of SMCC had 0.9 reactive group introduced per molecule. The IgG and F(ab') both were incubated with NlysPE40 or NlysPE38QQR overnight and purified on a size exclusion column. The fractions containing the 1:1 conjugates of the toxin with either the antibody or the F(ab') fragment represented 26% of the starting material for F(ab')-PE40 and 8% for IgG-PE40. Thus, the process of conjugation of the F(ab') fragment to the toxin is more efficient than conjugation to IgG under above-described conditions (Table 1).

Table 1 *Comparison of di- and monovalent immunotoxins containing HB21 and NlysPE40*

	IgG-PE40	F(ab')-PE40[a]
Recovery of $F(ab')_2$ fragments (%)		86
No. of thiols after derivatization or reduction (per molecule)	0.4	3.0
Steps in conjugate purification	2	1
Recovery of purified immunotoxin[b] (%)	8	24
Cytotoxic activity on human cancer cells (ID_{50} = ng/ml)[c]	3.0 (15.9)[d]	0.5 (5.3)
Immunoreactivity (% of HB21)	25	100

[a] Calculation of the recovery is relative to the content of $F(ab')_2$ fragments in an IgG; *i.e.*, it represents 77% of the whole IgG mass.
[b] Final recovery *versus* the sum of starting amounts of the antibody and toxin.
[c] ID_{50}, concentration at which isotope incorporation falls by 50% compared to nontreated cells.
[d] Numbers in parentheses, concentration (pM).

Fig. 2 shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis performed under both nonreducing and reducing conditions, and immunoblots of the conjugates. The IgG (M_r 150,000) digested with pepsin produces $F(ab')_2$ fragments (M_r 110,000). Reduced IgG reveals two bands: a M_r 50,000 heavy chain band and a M_r 25,000 light chain band. Reduction of the $F(ab')_2$ produced M_r 30,000 and M_r 25,000 bands which correspond to the truncated heavy chain and the whole light chain, respectively. NlysPE40 appeared as a single entity under both nonreducing and reducing conditions (Fig. 2). F(ab')-PE40 (M_r 95,000 protein on a nonreducing gel) is the product of a noncleavable linkage between the heavy chain of F(ab') and NlysPE40 with the light chain coupled to the heavy chain by a disulfide bond (Fig. 1*B*). Therefore, this conjugate is represented by a M_r 70,000 band plus a M_r 25,000 band of the light chain under reducing conditions. There were also minor bands of molecular weights of 60,000–70,000, indicating that the light and the heavy chains may form aggregates during the experimental procedure, and that a very small amount of the uncleavable conjugate between NlysPE40 and the thiol groups which participate in the formation of an interchain disulfide bond can be produced. Immunoreactivity of Western blot with a polyclonal antibody to PE revealed the bands corresponding to F(ab')-PE40 and NlysPE40. The same pattern of the gels was obtained with conjugates

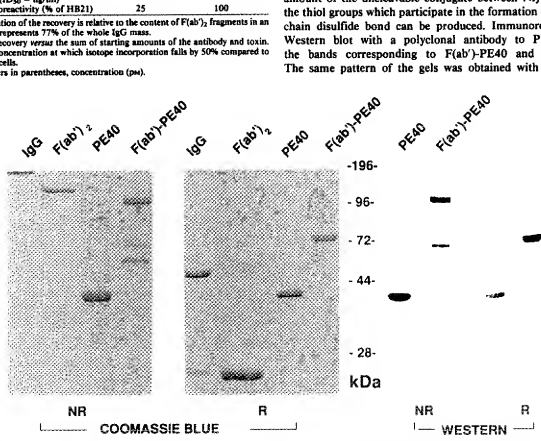

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of IgG, $F(ab')_2$, NlysPE40, and F(ab')-PE40 performed under nonreducing (*NR*) and reducing (*R*) conditions. Note that an identical pattern was obtained with NlysPE38QQR and its monovalent conjugate.

containing NlysPE38QQR (not shown). Thus, PE derivatives are coupled to a specific reactive site on the F(ab') fragment of HB21.

Cytotoxic Activities and Binding Affinities of Di- and Monovalent ITs. Since monovalent and divalent ITs are of different molecular sizes, the results will be expressed on a molar basis. However, it should be kept in mind that the change in valency may be important for antibody binding to an antigen. Therefore, the results based on a weight, which also happens to reflect a relative valency, will also be presented.

The cytotoxic activities of IgG- and F(ab')-ITs were evaluated by using protein synthesis inhibition assay on Colo205 and A431 carcinoma cell lines. Fig. 3 shows the results of a typical assay in which the cells were incubated with various concentrations of ITs. On Colo205 cells, the F(ab')-PE40 conjugate was extremely cytotoxic with a concentration at which isotope incorporation falls by 50% when compared to untreated cells of 5.3 ± 2.1 pM (0.52 ± 0.2 ng/ml; $n = 12$; SD). The IgG conjugate was 3-fold less active (15.9 ± 5.6 pM; 3.1 ± 1.1 ng/ml; $n = 5$; $P < 0.000001$ in Student's t test). To determine if the monovalent ITs are also more active on other cell lines, we carried out experiments on A431 cells and found that F(ab')-PE40 was several times more active than IgG-PE40 (data not shown). We also found the F(ab')-PE38QQR to be more cytotoxic than IgG-PE38QQR on A431 cells (Table 2). In addition, the F(ab')-PE38QQR was the most active agent tested. Thus, NlysPE40 and NlysPE38QQR conjugated to F(ab') are about 3 times more active ITs than IgG-ITs toxins.

We considered the possibility that F(ab')-PE40 and F(ab')-PE38QQR are more active than the divalent ITs because they bind more efficiently to the transferrin receptors. Therefore, we evaluated the binding of ITs on Colo205 cells as described in "Materials and Methods." The differences between the binding abilities of IgG, IgG-PE40, and F(ab')-PE40 were determined by an analysis of the corresponding displacement curves (Fig. 4). The IgG alone competed with a concentration required to inhibit the binding of labeled HB21 by 50% of 1.5×10^{-8} M ad F(ab')-PE40 displaced ^{125}I-HB21 equally well. In contrast, IgG-PE40 had a lower affinity for transferrin receptors than the IgG alone and F(ab')-PE40 (Fig. 4*A*). We repeated the experiment using F(ab')-PE40 and IgG-PE40 (Fig. 4*B*) and found that the concentration required to inhibit the binding of labeled HB21 by 50% for F(ab')-PE40 was 3 times lower (1.3×10^{-8} M) than that for IgG-PE40. These experiments demonstrate that using HB21 F(ab') fragments instead of the whole IgG for conjugation with NlysPE40 yields an IT with a higher affinity for the antigen, and its affinity is equal to that for the antibody alone (Table 1).

Table 2 In vitro *and* in vivo *activity of HB21 conjugates containing NlysPE38QQR*

Conjugate	Cytotoxicity on A431 cells (ID_{50} = ng/ml)	Toxic dose[a] pmol (μg)/mouse	Minimal effective dose pmol (μg)/mouse	Therapeutic index (toxic/minimal dose)
IgG-PE38QQR	0.5	52 (10)	26 (5)	2
F(ab')-PE38QQR	0.1	210 (20)	26 (2.5)	8

[a] A dose causing significant decrease in body weight.

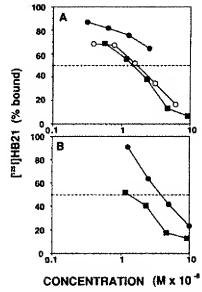

Fig. 4. The ability of IgG (○), IgG-NlysPE40 (●), and F(ab')-PE40 (■) to displace the binding of ^{125}I-HB21 to Colo205 cells. ^{125}I-HB21 had a specific activity of 20 mCi/mg and 40,000 cpm of bindable labeled antibody were added into the assay. *Points*, average of four determinations. *A* and *B* were performed separately.

Antitumor Effect of F(ab')-PE38QQR in Nude Mice. To evaluate whether F(ab') linked to recombinant mutants of PE may be useful therapeutically, we performed animal studies on nude mice bearing A431 tumors (Fig. 5). We used three groups of animals which were given injections i.p. of: (*a*) 0.2% human serum albumin/phosphate-buffered saline, (*b*) IgG-PE38QQR, and (*c*) F(ab')-PE38QQR, beginning on day 4 when small tumors had formed in all mice. The control group had tumors bigger than 600 mm^3 on day 12 when they had to be sacrificed (Fig. 5*A*; the end point in Fig. 6). In mice treated with 25 pmol (5 μg) or 52 pmol (10 μg) of IgG-PE38QQR per day, we observed a complete and sustained (45 days of observation) regression of A431 tumors in all the mice. Complete regression was also achieved by using F(ab')-PE38QQR. However, we noted two important advantages in using the monovalent IT. First, by day 7 the tumor regression seen with F(ab')-PE38QQR was significantly greater than that seen with IgG-PE38QQR, suggesting a faster and/or more potent antitumor

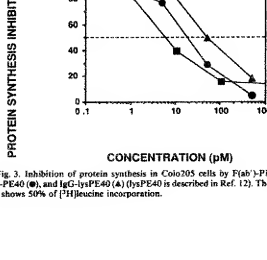

Fig. 3. Inhibition of protein synthesis in Colo205 cells by F(ab')-PE40 (■), IgG-PE40 (●), and IgG-lysPE40 (▲) (lysPE40 is described in Ref. 12). The *dashed line* shows 50% of [^{3}H]leucine incorporation.

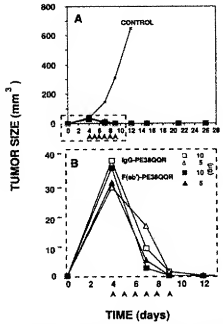

Fig. 5. *A*, antitumor effect of mono- and divalent ITs containing NlysPE38QQR in nude mice. A431 cells were implanted s.c. on day 0 and treated i.p. on the days indicated by the *arrowheads*. *B*, a magnified *dashed line* margined portion of (*A*). Mice received: (*a*) the vehicle (×); (*b*) IgG-PE38QQR: 5 or 10 μg/day, and (3) F(ab')-PE38QQR: 5 or 10 μg/day.

action than that of the divalent IT (Fig. 5*B*). Second, the monovalent conjugate was less toxic to animals (Fig. 6) than its counterpart composed of an IgG, as judged by the weight of animals and their appearance. There was no significant difference in body weight of animals on day 4 when the injections of ITs began. The mice which received 52 pmol (10 μg) and 105 pmol (20 μg) of the divalent and 210 pmol (20 μg) of the monovalent conjugate per day had the lowest body weights after therapy (Fig. 6*A*). In addition, the mice treated with 105 pmol (10 μg) per day of the monovalent conjugate were heavier after the treatment than those treated with 52 pmol (10 μg) per day of the divalent conjugate (Fig. 6*B*). This finding indicates that the mice had to be given injections of 4 times higher doses of the monovalent IT to match the toxicity of the divalent IT.

Since F(ab')-PE38QQR and IgG-PE38QQR completely eliminated A431 tumors in nude mice at a total dose of 30 μg, we used lower doses to determine the minimal amount of ITs necessary for tumor regression (Table 2). The treatment was started on day 4 after tumor implantation and carried out daily for 4 days. Both monovalent and divalent ITs were administered at doses of 2.5 and 1.25 μg/day for 4 days and the animals were observed for 2 weeks. IgG-PE38QQR exhibited a significant but incomplete antitumor effect at 12.5 pmol (2.5 μg) per day but not at 6.25 pmol (1.25 μg) per day. However, the monovalent IT caused complete regression of A431 tumors in all mice receiving 25 pmol (2.5 μg) per day and in 3 of 5 mice treated with 12.5 pmol (1.25 μg) per day. The injections of 6.25 pmol (0.625 μg) of F(ab')-PE38QQR still had a significant antitumor effect (data not shown) but they were ineffective when using the divalent IT (Table 2). Once again, we observed that the monovalent IT causes an earlier disappearance of tumors than the divalent compound. These results support the notion that the monovalent conjugate acts faster and perhaps more potently than the divalent IT.

DISCUSSION

Our present study was initiated to explore the properties of a monovalent IT in which either NlysPE40 or NlysPE38QQR was attached to the F(ab') portion of an antibody to the transferrin receptor through the heavy chain sulfhydryl groups contained within the hinge region. We demonstrated that these monovalent conjugates are more cytotoxic agents than ITs made using a whole antibody (Tables 1 and 3). Moreover, the monovalent ITs are extremely active antitumor agents in nude mice bearing solid tumors. Furthermore, independent of the molarity and weight or valency, F(ab')-PE38QQR has a 4 times better therapeutic index than IgG-PE38QQR (Table 3). Another advantage of the F(ab') containing IT is its high yield and ease of purification (Table 1).

The two F(ab')-ITs were severalfold more cytotoxic than IgG ITs on different cancer cell lines. The greater cytotoxic potency of F(ab')-ITs was unexpected in view of the reports on ricin A chain coupled to F(ab') fragments. Such conjugates were demonstrated to have lower cytotoxic activities than MAb-ricin A conjugates due to their diminished binding to the antigen (18, 25, 26). There are many factors that could influence the cytotoxicity of ITs and we tested the binding abilities of the monovalent and divalent ITs and compared them with the original antibody. F(ab')-PE40 had greater affinity for human

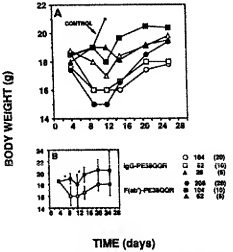

Fig. 6. Body weight of mice underwent treatment with mono- and divalent immunotoxins (*A*). The weight is expressed as mean and SD is included in *B*. Significant differences between the group receiving 10 μg (*B*) per day of the monovalent immunotoxin and the group receiving 10 μg per day of the divalent immunotoxin are indicated by * ($P < 0.05$).

Table 3 *Antitumor effect of low doses of the IgG- and F(ab')-PE38QQR conjugates*

		IgG-PE38QQR[a]		F(ab')-PE38QQR[a]	
		pmol (μg)/dose[b]			
Day	Control[a]	13 (2.5)	6.5 (1.25)	26 (2.5)	13 (1.25)
4	5/5	5/5	5/5	5/5	5/5
8	5/5	5/5	5/5	3/5	3/5
12	5/5	1/4	5/5	0/5	2/5
16	5/5	1/4	4/5	0/5	2/5
19	5/5	1/4	5/5	0/5	2/5

[a] Mean tumor size on day 4 was 40–50 mm³ (number of animals with tumors/number of animals implanted with tumors).

[b] Treatment started on day 4 after tumor implantation with four i.p. injections on consecutive days.

transferrin receptors than the conjugate with whole antibody (Fig. 4). It seems likely that an increase in binding contributes to the increased activity of the F(ab')-toxin. The main reason for the enhanced binding of the F(ab')-toxin in comparison to IgG-toxin is that in the F(ab')-toxin, the toxin molecules are located at one site, while in the IgG-toxin the toxins are present at many sites, including some close to the antibody-combining site. It is plausible that because of the spatial conformation of the ricin A chain, this toxin still interferes with the antibody-binding site when coupled to the F(ab') fragments.

In the present work we were able to determine the positions on NlysPE38QQR at which coupling could occur. This form of the toxin has only two amino groups available with which cross-linking reagent can react and both are located in the amino portion of the toxin. This region is removed by proteolysis within the cell to allow domain III to be translocated efficiently into the cytosol (Refs. 10 and 16; Fig. 1).

F(ab')-PE38QQR was an extremely efficient antitumor agent capable of causing complete and sustained tumor regression with lower toxicity than that observed with administration of the divalent conjugate. These data are in accordance with findings made by Fulton *et al.* (27) who demonstrated that monovalent ITs containing ricin A chain were significantly less nonspecifically toxic to animals than in the divalent versions. It is of interest that IgG-PE38QQR caused a complete regression of A431 tumors at much lower doses than IgG-lysPE40 used in the previous study (154 pmol; 30 μg *versus* 1020 pmol; 200 μg of a total dose, respectively; Ref. 12). The advantage in using PE derivatives without the lysine residues in domain III (NlysPE40QQR and NlysPE38QQR) for production of more active chemical conjugates has been recently documented with other MAbs, such as B3 and C242 (16).[6]

Another potential advantage of F(ab') containing ITs is their small size. F(ab')-PE40 or F(ab')-PE38QQR has a molecular mass of 95 kDa, whereas IgG-PE40 has one of 190 kDa. It has been shown that a $F(ab')_2$ (110 kDa) and/or F(ab') (55 kDa) fragments penetrate into different tumors much better than whole antibodies (28, 29). The same applies to the immunotoxins made up of fragmented antibodies (19, 27). Thus, F(ab')-ITs containing PE derivatives should be able to penetrate into a tumor with better efficiency than IgG-ITs. While our data support that the monovalent immunotoxin has a faster antitumor action than its divalent counterpart, we cannot indicate with certainty whether the better antitumor action of the monovalent toxin is due to its smaller size or to its higher cytotoxic potency. It is also important that $F(ab')_2$/F(ab') fragments do not interact with the Fc receptors present on many cell types, thus enhancing the availability and specificity of ITs (30). Finally, the thioether linkage used to couple F(ab') and PE has been found to provide a superior stability of such IT in the circulation than the one which would have a disulfide linkage (31). Since antibodies differ in their susceptibility to proteolytic cleavage it is important that F(ab') molecules can be now produced and reconstituted by recombinant techniques (32).

In summary, this study indicates that immunotoxins composed of the F(ab') fragment of monoclonal antibodies and NlysPE38QQR should be pursued as candidates for the treatment of human cancer.

ACKNOWLEDGMENTS

We thank E. Lovelace and A. Harris for assistance with tissue cell culture, T. Prior for reading the manuscript, and all other colleagues in LMB for comments, D. FitzGerald and C. Fryling for HB21 antibody, and A. Jackson, J. Evans, and P. Andryszak for typing the manuscript.

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